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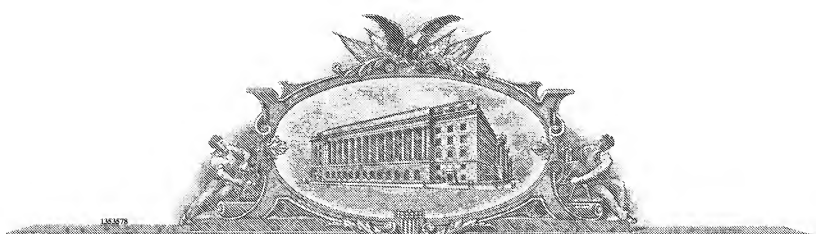

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APPLICATION NUMBER: ***60/559,802***
FILING DATE: ***April 06, 2004***
RELATED PCT APPLICATION NUMBER: ***PCT/US05/11626***

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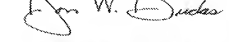

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. ER 651561805 US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Joseph R.	GARLICH	328 West Columbine Lane Westfield, Indiana 46268

Additional inventors are being named on the one *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

PTEN Inhibitors and Compositions for Therapeutic Utility in Treating Disease in Mammals

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☑ Customer Number: 22930

OR

☐ Firm or Individual Name					
Address					
Address					
City		State		Zip	
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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 29 ☐ CD(s), Number

☐ Drawing(s) *Number of Sheets* ☐ Other (specify)

☐ Application Data Sheet. See 37 CFR 1.76

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☑ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check or money order is enclosed to cover the filing fees.

☑ The Director is herby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 08-3038

☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE Amount ($): 80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME Teddy E. Scott, Jr., Ph.D.

TELEPHONE (312) 846-5621

Date April 6, 2004

REGISTRATION NO. 53,573 *(if appropriate)*

Docket Number: 01656.0006.PZUS01

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Docket Number 01656.0006.PZUS01

INVENTOR(S)/APPLICANT(S)

Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Donald L.	DURDEN	8820 Woodacre Lane Indianapolis, Indiana 46234

Number 2 of 2

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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 80.00

Complete if Known	
Application Number	
Filing Date	April 6, 2004
First Named Inventor	Joseph R. Garlich
Examiner Name	
Art Unit	
Attorney Docket No.	01656.0006.PZUS01

METHOD OF PAYMENT *(check all that apply)*

☐ Check ☐ Credit card ☐ Money Order ☐ Other ☐ None

☑ Deposit Account:

Deposit Account Number: 08-3038

Deposit Account Name: Howrey Simon Arnold & Wh

The Director is authorized to: *(check all that apply)*

☑ Charge fee(s) indicated below ☐ Credit any overpayments

☑ Charge any additional fee(s) or any underpayment of fee(s)

☐ Charge fee(s) indicated below, **except for the filing fee** to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below		Fee Paid
Total Claims		-20** =		X	=
Independent Claims		- 3** =		X	=
Multiple Dependent					=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) -0-

***or number previously paid, if greater; For Reissues, see above*

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for ex *parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) -0-

SUBMITTED BY				(Complete *(if applicable)*)	
Name (Print/Type)	Teddy C. Scott, Jr., Ph.D.	*Registration No. (Attorney/Agent)*	53,573	*Telephone*	312-846-5621
Signature				*Date*	April 6, 2004

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Attorney Docket No. 01656.0006.PZUS01

PROVISIONAL PATENT APPLICATION

OF

Joseph R. Garlich
(Westfield, IN)

and

Donald L. Durden
(Indianapolis, IN)

FOR

PTEN INHIBITORS AND COMPOSITIONS FOR THERAPEUTIC UTILITY IN TREATING DISEASE IN MAMMALS

Following are attached ideas and a grant application outlining how to make inhibitors of PTEN for therapeutic use in mammals.

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. DO NOT EXCEED THE SPACE PROVIDED.

Important cellular processes are controlled by cycles of phosphorylation and dephosphorylation involving lipids and proteins. PTEN (phosphatase located on chromosome 10) is a dual specificity phosphatase which dephosphorylates an important lipid second messenger, phosphatidylinositol 3,4,5 phosphate [PtIins(3,4,5)P3] to control cell division and apoptosis.

Recent data has surfaced which supports our hypothesis that PTEN inhibition will modulate the apoptosis response under conditions of stress. Therefore an agent which would inhibit PTEN thereby augmenting levels of PIP3 would be likely to have therapeutic efficacy in a number of disease states associated with uncontrolled cell death and tissue damage. For example PTEN inhibitors could be a major advance in protecting marrow from the debilitating effects of chemotherapy and radiation therapy. However, no effective small molecule inhibitors of PTEN are known.

The crystal structure of PTEN has recently been reported with a tartrate molecule in the active phosphatase pocket. Based in this crystal structure data and our own preliminary molecular modeling we have hypothesized several classes of potential PTEN inhibiting organic molecules. We propose the combinatorial chemistry-based preparation of focused libraries of these potential PTEN inhibitors coupled with a suitable throughput PTEN bioassay in an effort to identify potent drug-like PTEN inhibitors.

PERFORMANCE SITE(S) *(organization, city, state)*

ComChem Technologies Inc.
8496 Georgetown Road
Indianapolis IN 46268

Indiana University Medical Group
James Whitcomb Riley Hospital for Children
702 Barnhill Drive, Room 2720
Indianapolis, IN 46202-5225

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Joseph R. Garlich, Ph.D.	ComChem Technologies Inc.	Principal Investigator
Donald L. Durden, M.D., Ph.D.	Indiana University Medical School	Co-Investigator
TBA, Ph.D.	ComChem Technologies Inc.	Senior Research Scientist
TBA, M.S.	ComChem Technologies Inc.	Research Scientist
TBA, Ph.D.	Indiana University Medical School	Post-Doc Biochemist

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☒ Yes ☐ No

RESEARCH GRANT

TABLE OF CONTENTS

RESEARCH PLAN

A. Specific Aims

1. Use molecular modeling to design and select potential library inputs.
2. Use solid-phase chemistry to access focused target designed libraries of PTEN inhibitors.
3. Evaluate libraries in medium throughput assay for PTEN inhibition.
4. The best selective PTEN inhibitors from 3 are evaluated for IC50 values.
5. The best compounds from Aim 4 are tested for PTEN in vitro inhibition selectivity.
6. Evaluate best compounds (modified as acetoxymethyl derivatives) from Aim 5 in cell based assays for functional PTEN inhibition.

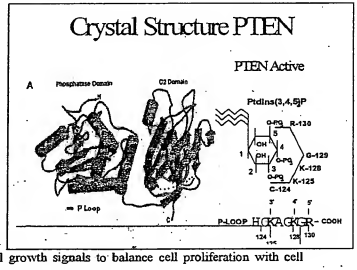
B. Significance

Background and Existing Knowledge- PTEN

Cellular processes are to some extent controlled by cycles of phosphorylation and dephosphorylation involving lipids and proteins. PTEN (phosphatase located on chromosome 10) is a dual specificity phosphatase which dephosphorylates an important lipid second messenger, phosphatidylinositol 3,4,5 phosphate [Ptlins(3,4,5)P3] to control cell division and apoptosis. It is mutated at high frequency in human malignant disease (incidence varies from 20% to 95% depending on tumor type). Preliminary data from the Durden group has implicated PTEN in the control of tumor-induced angiogenesis and the control of immunoreceptor signaling suggesting that this is a major drug target for control of angiogenesis and inflammatory signals.

Other laboratories have shown that PTEN exerts control of sensitivity of cells to stress induced apoptosis. More recently the Durden group demonstrated the first direct evidence that PTEN controls the activity of the nuclear tumor suppressor protein, p53. This suggests that

Figure 1. This shows crystal structure of PTEN with P-loop and amino acid sequence which composes the P-loop and its predicted interactions with the phosphoinositol ring. The 3 highly conserved basic residues in the P-loop, R, 130; K, 125 and K, 128 associate with the negatively charged phosphate groups in the phosphoinositol ring to coordinate catalysis at the D3 position mediated by conserved cysteine residue at position 124 which forms the critical thiol-phosphate intermediate required for catalysis [1].



PTEN coordinates cell sensitivity to cell growth signals to balance cell proliferation with cell death (apoptosis) and angiogenesis.

These data together with evidence in the literature support the hypothesis that PTEN inhibition will modulate the apoptosis response under conditions of stress. Such inhibitors would increase PIP3 levels and prevent programmed cell death and promote survival of important cell populations cardiac myocytes, neuronal cells, etc under conditions of genotoxic or environmental stress e.g. hypoxia. Such inhibitors would serve to salvage tissue under conditions where stressful stimuli would tend to destroy normal organ functions. Therefore an agent which would inhibit PTEN thereby augmenting levels of PIP3 would be likely have therapeutic efficacy in a number of disease states associated with uncontrolled cell death and tissue damage. In particular

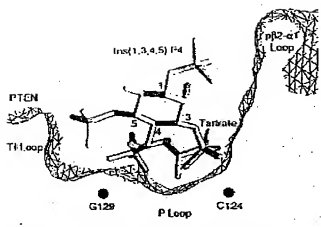
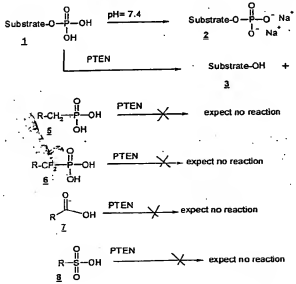


Figure 2. Model of Ins(1,3,4,5)P4 Binding to the PTEN Active Site
The phosphate groups at the D3 and D4 positions of Ins(1,3,4,5)P4 are superimposed on the crystal structure derived carboxylate groups of the tartrate molecule bound in the PTEN active site. Note the extended pocket unique to PTEN on the left side into which the D5 phosphate group protrudes.

we believe the delivery of such inhibitors to the bone and bone marrow could be a major advance in protecting marrow from the debilitating effects of chemotherapy and radiation therapy.

The crystal structure of PTEN has recently been reported with a tartrate molecule in the active phosphatase pocket [1]. The overall structure of PTEN is shown in Figure 1 along with the structure of its substrate, Ptlins(3,4,5)P3. The superimposition of phosphate groups at the D3 and D4 positions of Ins(1,3,4,5)P4 with those of the carboxylate groups of the tartrate found in the crystal structure is shown in Figure 2, reproduced from the literature [1]. The PTEN active site pocket is about 8 angstroms deep with an oval opening of about 5 by 11 angstroms. The left-side extension of the pocket in which the D5 phosphate protrudes (the T loop area) is unique among phosphatases and thus represents a critical target space for us to fill in order to prepare specific PTEN inhibitors. In fact, mutations at Gly129 in the T1 loop decrease the size of this extension and disrupts PTEN's PI(3,4,5)P3 phosphatase activity but allow for retention of tyrosine phosphatase activity (because tyrosine would not require this extention pocket space)[2,3].

Figure 3. Stable Monophosphate Ester Mimics



Background and Existing Knowledge-Phosphate mimic chemistry

Since phosphatases remove phosphate from substrates it is reasonable to suggest a mimic of a phosphoric acid monoester as a likely inhibitor since this group is at least one part of the molecule that fits well into the protein binding pocket. This is shown schematically in Figure 3 where PTEN removes the D3 phosphate of phosphatidylinositol 3,4,5 phosphate [Ptlins(3,4,5)P3] which is represented by structure 1 to give the alcohol of the substrate (3) and

ultimately inorganic phosphate (4). It should be noted that at physiological pH phosphates exist in highly ionized forms such as represented by structure 2. This high degree of ionization is taken advantage of by our synthetic target design but can represent a barrier to crossing cell membranes. This aspect is also addressed by our research plan. Researchers have proposed several mimics of the phosphate monoester such as carboxylic acids (7), sulfonic acids (8), and phosphonic acids (5) [4,5,6,7]. The key for an inhibitor is that the mimic group is ionizable, is about the same size as the phosphate group and contains no hydrolizable bonds. As depicted in Figure 3 all of these phosphate mimics fulfill this criteria. Of particular interest is the α, α difluoro-phosphonic acid (6) reported to be an excellent mimic of phosphonate monoesters due to loweing the phosphonate pKa2 and introducing hydrogen-bonding interactions similar to the ester-linking oxygen found in phosphonate monoester [6,7].

Commercial Opportunity for PTEN Inhibitors. At the end of this project period we will have several validated PTEN inhibitor candidates and will have shown how we can prepare prodrugs capable of crossing cell membranes. Additional funding will be obtained (such as Phase II SBIR or venture capital) to refine and optimize the performance for such an inhibitor. It is likely that selective targeting will be needed to minimize potential side effects of a systemically administered PTEN inhibitor. For example we aim to develop technology to deliver such inhibitors selectively to bone marrow for marrow protection purposes during chemotherapy and radiation therapy. The market potential for the products arising from this grant research, for example a bone marrow sparing agent for application in chemotherapy, could be as much as a $1 billion per year. This estimate is based on the current success for related products such as Amgen's Neupogen (>$1.3 billion in global sales in 2000) for restoring neutrophils to adequate protective levels in patient undergoing chemotherapy. The potential advantage of our approach would be a single injection prior to chemotherapy versus daily treatment of patients with Neupogen (now improved with the approval of weekly injections of Neulasta which is Amgen's PEGylated version of Neupogen (pegfilgrastim)). Additionally, with our approach the neutrophils should not even drop upon exposure to chemotherapy or radiation due to inhibition of PTEN induced apoptosis and thus the patients would not be at risk due to dropping neutrophils.

Importance of Proposed Research

Finding a potent inhibitor of PTEN is the first step in our quest to find an improvement in the treatment of cancer. Because bone marrow suppression is usually the dose limiting toxicity in many chemotherapeutic and radiation therapy regimes any approaches to mitigate the bone marrow suppression should result in more aggressive treatments with attendant increased anticancer effects. Our approach is that with a PTEN inhibitor in hand we can then use targeting technology to deliver this drug selectively to the bone marrow which will prevent bone marrow cells from undergoing apoptosis during aggressive cancer treatment. This regiment would be of utility to all cancers except those having a component residing in the bone or marrow. This type of approach is being pursued by others using the p53 inhibitor pifithrin, which inhibits apoptosis, with good results so far. Since PTEN controls the p53 signaling process we believe inhibiting PTEN will also effectively inhibit the activation of p53. Thus, our discovery of a PTEN inhibitor during this grant will allow for the testing of this hypothesis and then the groundwork is laid for a novel drug development aimed at protecting the bone marrow from the effects of chemotherapy and radiation therapy.

C Relevant Experience. One key meritorious point to consider in the current proposal is the bringing together of the Durden group's expertise in signal transduction, molecular biology, and oncology with the combinatorial and synthetic chemistry expertise, drug design and conjugation expertise found in the Garlich group to evaluate the feasibility of propelling conventional

treatments to the next level using late breaking science (cell signal understanding). This SBIR program grant is the perfect vehicle to make this interaction happen.

Principal Investigator; Dr. Garlich, CCTI Chief Scientist, has eleven years of industrial experience at Dow Chemical in the area of radiopharmaceutical discovery and development. He was instrumental in the synthesis and formulation development for ^{153}Sm-EDTMP, an FDA approved bone-seeking radioactive drug for the relief of bone pain associated with bone metastases, licensed to Cytogen Corp.(Quadramet™). These new molecular entities are phosphonic acid based drugs. He also developed new azamacrocycles (synthesis and new uses) as well as bifunctional chelating agents for conjugation to monoclonal antibodies. He is the father of ^{166}HoDOTMP, a bone-seeking polyaminophosphonic acid based radiopharmaceutical, now in phase III clinical trials for the treatment of multiple myeloma (licensed by Dow to NeoRX). More recently, before founding ComChem Technologies, he was responsible for establishing the combinatorial chemistry group at Dow AgroSciences and has experience in all aspects of combinatorial chemistry and parallel synthesis including automation, solid-phase and solution phase synthesis, analytical instruments and methodology. Additionally and relevant to this proposal, he has extensive experience in phosphonic acid chemistry (synthesis and purification and interactions in biological envrironments).
Co-Investigator (via subcontract with Indiana University): Dr. Durden, MD, Ph.D. has five years experience and expertise in vascular biology and the study of angiogenesis and integrin signaling. His laboratory recently discovered that PTEN and PI-3 kinase regulate tumor-induced angiogenesis[15]. He is an expert in the biochemical and molecular dissection of signal transduction pathways in mammalian cells. He will screen inhibitors pathways including defining specificity of signaling in different cell systems including tumor cells, myeloid cells, endothelial and neuronal cells and cell lines. Dr. Durden has significant experience in animal models for tumor progression, angiogenesis and the study of inflammation. He has ten years experience in cell biology, molecular biology and the biochemical study of protein and lipid kinases. In addition as a board certified Pediatrician and Pediatric Oncologist he will partieipate in the interpretation of the results obtained in the context of their potential for clinical application to cancer and inflammatory diseases.

D. Research Plan Experimental

A. Specific Aims

Specific Aim 1. Use molecular modeling to design and select potential library inputs.
We have performed preliminary modeling using BioMedCaChe 5.0 to manually dock a few members of the proposed (Aim 2) library to make sure the scaffold (various tyrosine analogs) will fit into the cavity and to estimate the size of the binding pocket. We will examine these assumptions and findings further as we choose the various inputs to attaché to the scaffolds from what is available commercially or what we could make easily. Also, when results are known from the initial screening we can use some basic modeling to try to discern patterns and help us devise a follow-up focused library hypothesis.

Specific Aim 2. Use solid-phase chemistry to access focused target libraries of potential PTEN inhibitors.

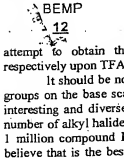
Specific Aim 2: Section A. General Approach.
Based on the crystal structure attributes as described above in the background section one critical aspect of our inhibitor design will be to prepare tyrosine-phosphate derivatives substituted such that part of the molecule would physically occupy the T loop pocket extension space and hence be specific for PTEN and not other tyrosine phosphatases. - An additional difference between PTEN and other protein tyrosine phosphatases is the presence of two basic groups, Lys-125 and Lys-128, in the center of the P-loop (Figures 1,2). These basic groups (protonated underphysiological conditions) give rise to the attraction of the highly anionic PI(3,4,5)P3

substrate. This information thus serves as our second point of molecule design which is to include P-loop lysine binding moieties. These would include acidic groups such as carboxylate, phosphate, phosphonate, sulfonate, hydroxamate, phenolate, and the like which are capable of bearing an negative charge at physiological pH. It should be kept in mind that PTEN has been demonstrated to dephosphorylate tyrosine-, serine-, and threonine-phosphorylated peptides as well as phosphatidyliniositol (3,4,5)-trisphosphate (PI(3,4,5)P3) with specificity for the D3 position of inositol). Our effort in this proposal toward devising a PTEN inhibitor focuses on resembling the size and shape of PI(3,4,5)P3 and phosphorylated tyrosine. Since the structure of these substrates in the binding pocket of PTEN isn't known we are using modeling to get us in the ball park and using combinatorial library production to explore certain regions of chemical space to find a lead inhibitor molecule. Our approach is to make polyanionic tyrosine-like molecules that will fit into the binding pocket of PTEN and enter into additional stabilizing binding to the peptide and thus inhibit its dephosphorylation of ability. Our approach is to use known chemistry, specifically unnatural peptide synthesis, applied to new molecules to prepare PTEN inhibitor target libraries based on a tyrosine-like scaffold..

Specific Aim 2: Section B. Solid Phase Synthesis of Potential PTEN Inhibitors using Tyrosin-like Scaffold

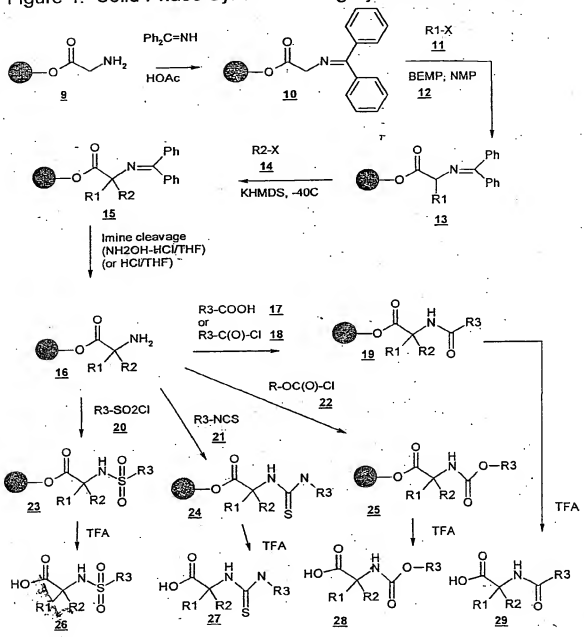
Briefly, the library synthesis is shown in Figure 4 and will start with commercially available Fmoc-protected Wang resin (the dark circles represent the polymeric styrene-divinylbenzene crosslinked solid-phase synthesis resin) which is made ready for use by exposing to piperidine to yield the starting glycine-resin (**9**). Exposure to commercially available benzophenone imine (1.5 equiv) in N-methylpyrolidinone (NMP) with acetic acid (1.3 equiv) overnight at room temperature should give complete conversion to the activated resin bound imine **10** [17] . The resin is then exposed to 2 equivalents of the nonionic "Schwesinger Base" [18], BEMP (see structure in Figure 5) to generate the nucleophilic resin bound anion and exposed to 2 equivalents of the first alkylating agent, **11**, in NMP at room temperature overnight. Washing of the resin will yield the resin bound **13** ready for the second alkylation. The second alkylation requires a much stronger base such as potassium hexamethyl disylazane (KHMDS) as described in the literature [19] and has been shown to react with a variety of alkyl halides working particularly well with benzylic halides [J3,J4]to yield the α, α-disubstuted resin bound aminoacid, **15**. The imine protecting/activating group is then removed under neutral conditions with hydroxylamine hydrochloride or under mildly acidic conditions with hydrochloric acid in THF to give the free amine bound to resin, **16**. This amino group, even with two bulky α, α-substituents is reported in the literature to be readily acylated using acid chlorides or carboxylic acids with coupling agents [19,21] to give acylated resin construct **19**. Routine cleavage of amino-acids bound to resin is then accomplished with trifluoroacetic acid to give the final α, α-disubstuted product, **29**, having 3 points of substitution arising from two alkylations and an acylation. This chemistry path is well explored and will be our main library production focus. However, we are also planning on reacting the resin-bound free amino group of **16** with sulfonyl chlorides (**20**), isothiocyanates (**21**), and chloroformates (**22**) to attempt to obtain the corresponding sulfonamides (**26**), thioureas (**27**), carbamates (**28**) respectively upon TFA cleavage.

Figure 5



It should be noted that from a combinatorial perspective our ability to put three different groups on the base scaffold could result in huge numbers. For example, there are at least 100 interesting and diverse acids and acid chlorides commercially available and probably an equal number of alkyl halides which are possible synthetic inputs which would yield a 100X100X100= 1 million compound library. We are not set up for such a large scale production nor do we believe that is the best way to find a PTEN inhibitor. Instead we have taken into account the

Figure 4. Solid Phase Synthesis using Tyrosine-like Scaffold



information from the crystal structure of the binding cavity, coupled with medicinal chemistry insight into diversity and our knowledge of solid phase synthesis chemistry to design a focused set of compounds to make. A listing of 5 inputs from each group of the three "acylating" reagents is shown in Table 1 for representative purposes only. All of these compounds are available commercially.

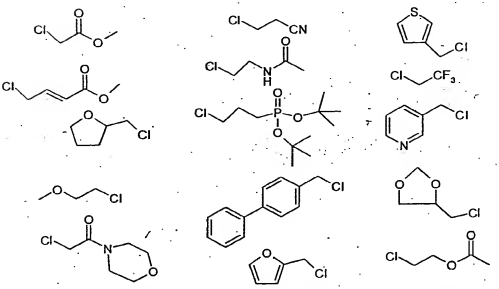
Table 1:
List of Available Acids, Acid Chlorides, and Sulfonyl Chlorides as Possible Library Inputs

R3-COOH (17)	R3-C(O)-Cl (18)	R3-SO2Cl (20)
		
		
		
		
		

The limited number of isothiocyanate inputs (21) that we will use (about 10)would be prepared from commercially available primary amines reacted with thiophosgene under basic conditions. The limited number of chlorformates (22) that we will use (about 10) will be prepared from reacting commercially available primary alcohols with triphosgene.

The initial input in the library synthesis is R1-X and is required to be a primary alkyl halide and commercially available. A partial list of 15 candidates we are considering for this library and that are commercially available is shown in Figure 6. These input groups are chosen for their varying ability to hydrogen bond (accept and donate), to hydrolyze to acidic functionality, to be lipophobes, and to be lipophiles.

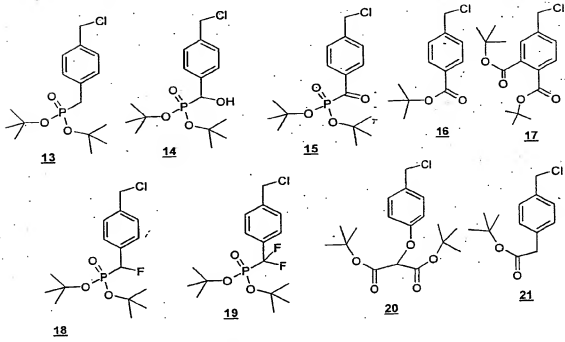
Figure 6. List of Commercially Available R1-X



The critical piece of our focused library design is the R1-X input which is the unit that will imitate the tyrosine-phosphate ring-phosphate ester moiety and will likely make the most important binding contribution. Examples of the R2-X inputs are shown in Figure 7 and they are not commercially available. Note that these are all benzylic halides which should make excellent alkylating agents as the R2-X input in Figure 4. Note also that these inputs all have tertiary butyl esters which will be cleaved under the final conditions to cleave the compounds from the solid phase resin. Alternatively, we envision simple alkyl phosphonate ester protecting groups such as methyl or ethyl which can be cleaved while the compounds are on the resin by exposure to trimethylsilyl bromide prior to trifluoroacetic acid cleavage. It should be noted that we intend to synthesize one compound per well by using a spatial array combinatorial approach but that in all cases there is at least one chiral center created with the first alkylation step. This center, because of the chemistry will be racemic. Thus in the best case our cleaved compounds will have two enatiomers in each well. Additional chirality is introduced in some of the other inputs such as **14** and **18** so that these compounds upon cleavage from the resin will exist as several stereoisomers (2^n where n is the number of chiral centers in the molecule). As a screening library this is a reasonable approach with hit followup being a chiral column chromatography step to separate the isomers and find out if the activity belongs to one certain stereoisomer.

All of the important inputs shown in Figure 7 will need to be synthesized. There is ample literature precedent for the various steps we propose to get to the final target input structures. For example, in Figure 8 is shown the synthesis of **19** as taken exactly from the literature [22]. The synthesis starts with commercially available 4-chloromethylbenzoyl chloride (**22**) reacting at low temperature with trivalent phosphite ester to give the target compounds alpha-keto phosphonic acid **15**. This compound is then reacted with DAST (diethylaminosulfur trioxide) to replace the keto with an alpha difluoro motief to give target alkylating agent **19**. Also, final target **15** can be reduced with sodium borohydride at low temperature to give the alpha hydroxyl target phophonate **14**. Target **14** likewise reacts with DAST to give the mono-fluoro phosphonate target **18**. All of these are reported to occur (for the case where R= ethyl ester group) from 50 to 90% theorectical yield [22].

Figure 7. Synthesized Inputs for R2-X; Tyrosine-phosphate mimics

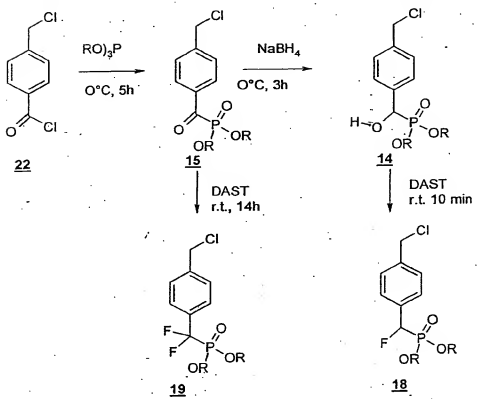


Using similar published literature precedent [25,26,27] we will make the other target alkylating agents listed in Figure 7.

Putting the various components together (again emphasizing that we are setting up one compound per well parallel synthesis of all possible combinations of our selected inputs) will generate a total of about 3000 compounds. This comes from about 15 R1-X reacted with about 25 total "acylating" agents (including sulfonylation, reaction with isothiocyanates and chlorformates) and alkylated with the 9 synthesized R2-X compounds of Figure 7 for a numeric total of 15X25X9X=3373 compounds. The libraries will be produced at a reasonable production rate over the last two thirds of the budget period with the first two months being used to prepare the target intermediates of Figure 7. To give the reviewers a feel for the final target structures we present three examples of final targets derived from different inputs from the tables and cleaved from resin and shown in Figure 9.

Since PTEN inhibition is to take place inside a cell for therapeutic purposes there is a fundamental barrier to using small charged molecules (such as phophonates). However, there has been some work recently on using acetoxymethyl groups as a physiologically reversible protecting group to allow polyphosphonates to penetrate cell membranes [23,24,28]. This is shown using chemical structures in Figure 10 using commercially available bromomethyloxyacetate. Thus, a limited number of leads from the libraries described above can be converted to such phosphonate (and/or carboxylate) derivatives in order to get into cells to inhibit PTEN as needed for the cell based assay described in Aim 6.

Figure 8. Literature Synthesis of Key Alkylating Agents **14**, **15**, **18**, **19**



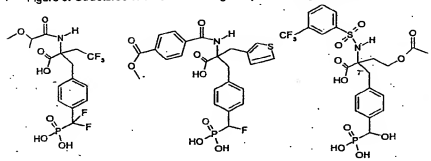
Specific Aim 3. Evaluate libraries in medium throughput assay for PTEN inhibition.

The combinatorial libraries (1 compound per well) will be screened for PTEN inhibition using purified recombinant PTEN phosphatase in a 96 well high throughput format using a colorimetric assay based on the detection of inorganic phosphate released from phosphopeptides or synthetic phospholipid substrates *in vitro*. Liberated inorganic phosphate is detected in twenty microliters of supernatant using the Malachite green assay and an inorganic phosphate standard curve. Malachite green reaction with inorganic phosphate is detected spectrophotometrically at 620 nm wavelength [8]. A series of endpoint reaction data points can be used to construct a kinetic curve representing PTEN enzymatic activity. The threshold for considering positive effect of candidate compounds for anti-PTEN activity will be 20% inhibition. This benchmark can be adjusted to give a manageable number of compounds to follow up with in the assays noted below. It should be noted that the Durden group has extensive experience with this assay and thus no significant development time is needed to get it established. We expect to be able to run this assay in such a manner as to keep up with the compounds generated in Specific Aim 2.

Specific Aim 4 The best selective PTEN inhibitors from Specific Aim 3 are evaluated for IC50 values.

Compounds showing PTEN inhibition >20% as describe in Aim 2 will then be examined at various concentrations to determine IC50 values. Linearity for this assay is established for each screening run in the absence and presence of known phosphatase inhibitors. Positive controls for inhibitory activity will include oxidizing agents, vanadinium ions and phenylarsine oxide and para nitrophenylphosphate as competitive and irreversible inhibitors of phosphatase activity (13, 14). We hypothesize that using the libraries proposed herein we will discovery PTEN inhibitors with IC50 values of at least as good at 5 uMolar. A 5 uMolar hit can then be optimized using the methodology set forth in specific aim 2 using inputs aimed at obtaining SAR information (and not diversity as in the initial libraries).

Figure 9. Structures of Three Final Targets by Combining Different Inputs



23 24 25

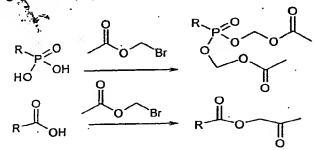
Specific Aim 5 The best compounds from Aim 4 are tested for PTEN in vitro inhibition selectivity.

Towards the end of the project period all of the compounds found to have IC50 values of 5 uMolar or better will be further examined to determine if they possess the desired selectivity for PTEN inhibition versus other phosphatases. To determine specificity the Durden group will then evaluate these compounds for inhibitory effects on other protein phosphatases including the 5' phosphatidylinositol phosphatase, SHIP [15] and a number of recombinant protein tyrosine phosphatases under study in the Durden laboratory to include SHP-1, SHP-2, PTP1B and PTP [9-12]. In this manner we hope to isolate small molecule inhibitors which target specifically the PTEN active site and not other lipid or tyrosine phosphatases.

Specific Aim 6. Evaluate best compounds (modified as acetoxymethyl derivatives) from Aim 5 in cell based assays for functional PTEN inhibition.

[Note; for this test the limited number of test compounds will be derivatized using the chemistry outlined in Figure 10]

Figure 10. Lipophilic Prodrugs of Phosphonate and Carboxylate



A critical measure of success for this technology is that the PTEN inhibitors must be able to cross cell membranes to exert their effects intracellulary. Thus, the handful of compounds (we

estimate <10) with the best selectivity and best IC50 values will be evaluated for their ability to functionally inhibit PTEN in cell-based assays. The Durden group has an established cell-based assay based on an immunoreceptor signaling pathway in COS7 cells which is under the negative control of PTEN. The capacity to block PTEN would be measured by augmentation in immunoreceptor signaling as measured by increased Fc receptor mediated phagocytosis. This assay measures the inhibitors ability to suppress PTENs capacity to inhibit phagocytosis in heterologous COS cell system [16]. Briefly, COS7 cells are cotransfected with cDNA constructs required for IgG mediated phagocytosis of opsonized sheep red blood cells in presence of absence of PTEN cotransfection. PTEN transfection completely abrogates phagocytosis in this assay system. Therefore candidate inhibitors of PTEN will be tested in this system for inhibitory effects on PTEN as measured by the uptake of red blood cells by phagocytosis. The Durden group will also test confirmed PTEN inhibitors in this phase for capacity to block PTEN induced arrest in integrin directed migration in a well established PTEN reconstituted tumor cell line [15]. Briefly, the Durden group has been able to completely abrogate integrin directed migration in U87MG cells by the stable and inducible expression of PTEN. This migration system will be used as an additional cell based assay for inhibitors of PTEN. PTEN inhibition will induce augmented integrin directed migration in this genetically determined system of PTEN suppression. In addition, cell based functional assays which are regulated by SHP-1 are under study in the Durden laboratory and could serve to confirm specificity in cells. These cell-based assays are all available in the Durden laboratory [16]. It should be noted that at this stage there will be only a handful of the best compounds. In order to perform cell-based assays we will likely have to convert the highly anionic inhitors (such as those containing a phosponate group) to a masked analog capable of crossing the cell membrane. We will do this by converting all the anionic groups to neutral readily hydrolizable masking groups as shown in Figure 10. Briefly, exposure of the inhibitor to the commercially available bromomethyleneacetoxy derivatizing reagent under mild basic conditions followed by reverse phase preparative HPLC will give substantially pure nonionic prodrugs as shown in Figure 10.

Summary/Milestones for Phase I

1) At least 5 target inputs from Figure 7 are prepared in multigram quantities.
2) Solid phase synthesis methodology taken from the literature (unnatural peptide synthesis) had been applied to the three input library scheme of Figure 4 to give at least 2000 compounds.
3) All compounds from #2 have been tested in initial screens.
4) Inhibitors showing >20% inhibition of PTEN (can be adjusted based on screen performance to give a manageable number of hits) have been tested in our PTEN IC50 assay.
5) Compounds with IC50 better than 10uMolar are tested for phosphatase specificity inhibition.
6) Compounds showing phosphatase specificity inhibition have been converted to nonionic prodrugs and tested in a whole cell assay.

Brief Glimpse into Phase II SBIR

The results of phase I, which will include one or more potent PTEN inhibitor lead candidates, will allow us to plan out the development of such inhibitors for therapeutic purposes. In phase II we will optimize the activity and develop drug targeting strategies depending on the molecular structure. Drug targeting will likely be necessary because of the vital role PTEN plays in so many signaling processes. In particular we will propose an initial application whereby PTEN inhibitors can be delivered selectively to the bone marrow where they can inhibit apoptosis as a side effect of radiation and chemotherapy treatment of cancer. This would allow for higher dose treatments since the bone marrow if often the dose-limiting toxicity. Animal studies in normal and diseased animals are envisioned to determine pharmacokinetic profiles, metabolism, excretion and efficacy are all planned for phase II to move this technology towards phase I/II human clinical trials.

E HUMAN SUBJECTS- NONE

F VERTEBRATE ANIMALS-NONE

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H CONTRACTUAL ARRANGEMENTS- Upon receiving funding for this SBIR proposal the collaboration between CCTI and Dr. Druden's lab at the Indiana University Medical School will be formalized with a research contract. The budgetary considerations of this arrangement are outlined in Dr. Durden's support letter at the end of this proposal. Basically this contract calls for a 5% commitment of Dr. Durden's time to help implement the PTEN assays and interpret results and pays for 100% of a post doc in his lab to organize, plan, implement, and run the biological assessment studies. This collaboration combines the combinatorial chemistry expertise of CCTI with the biochemistry and molecular biology expertise of Dr. Durden and his group.

I CONSULTANTS-NONE

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Disclosure Document Deposit Request

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Inventor(s): Joseph R Garrich

Title of Invention: Ideas on PTEN Inhibitors and Targeted Delivery thereof

Enclosed is a disclosure of the above-titled invention consisting of 3 sheets of description and ______ sheets of drawings. A check or money order in the amount of ______ is enclosed to cover the fee (37 CFR 1.21(c)).

The undersigned, being a named inventor of the disclosed invention, requests that the enclosed papers be accepted under the Disclosure Document Program, and that they be preserved for a period of two years.

Joseph R Garrich (signature)	
Signature of Inventor	Address
Joseph R Garrich	328 West Columbine Lane
Typed or printed name	
3-5-01	Westfield IN 46074
Date	City, State, Zip

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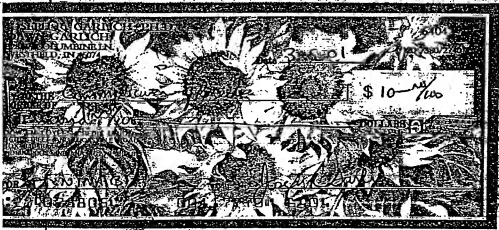


3-5-01

Inventor: Joseph R. Garbow Joseph R Garbow

Title: IDEAS ON PTEN INHIBITORS AND TARGETED DELIVERY Thereof

PTEN is a dual specificity phosphatase which dephosphorylates acidic peptides and inositol phospholipids. It has been identified as having a role in suppressing tumors. When mutated it appears to allow for the initiation and progression of malignant tumors such as glioblastomas. PTEN is reported to have a role in causing apoptosis (similar to p53). Thus inhibition of PTEN could cause a desensitization to chemotherapy or ionizing radiation. However, one must target it selectively or else you would be protecting the tumor also (unless the tumor was a mutant inactive form of PTEN).

I thus propose:

1) Inhibitors of PTEN having two acidic moieties that are anionic at physiological pH and relatively low molecular weight (< 700 daltons).

2) Inhibitors as in 1 that contain an additional appendage that would fit into the T1 loop (see Cell, 1999 p 323-334) region of the substrate phosphate binding pocket so as to confer specificity to PTEN for this inhibitor. This inhibitor would also take advantage of the two nearby PTEN lysines + arginine.

3) The use of inhibitors of PTEN applied to human skin to prevent chemotherapy or radiation

induced alopecia (hair loss). Such topically applied PTEN inhibitors would be formulated to optimize their delivery to hair follicles but not allow a significant amount of inhibitor to believe in the blood stream.

4) The use of PTEN inhibitors linked through a covalent cleavable (under physiological conditions) linker to a targeting moiety:

PI = PTEN inhibitor
CL = physiological cleavable linker
TA = Targeting agent

The CL can be groups such as imines, ketals, acetals, ureas hydrazones, esters, amides, etc which when appropriate substitutions can be cleaved under physiological conditions to generate the PTEN inhibitor ~~intact~~ = (or without only chemical trace of having been linked to the CL moiety). The TA moiety can be any organic group that confers target specificity such as specific peptide sequences (RGD) antibody or antibody fragments, receptor and molecules, liposomes or particles for organ or cell specific uptake, phosphonic acids, phosphates, amino phosphonic acids, acidic polypeptides or bone-attracting peptides all for targeting the construct to bone.

5) The use of PI-CL-TA constructs described in 4 which target the bone for sparing the cartilaginous marrow from the devastating effects of chemotherapy or ionizing radiation.

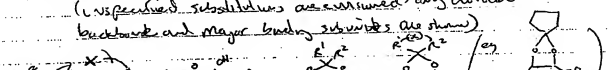
6) The use of PI-CL-TA which target cartilage in humans (various Quats) for treatment of Cartilage related diseases.
↳ quaternary ammonium compounds

3-5-01

Mentor: Joseph R. Garbe Joseph R. Garbe Page 3 of 3

cont'd

1) Specific Compound types proposed as PTEN inhibitors (conserved substituents as ascertained only cortical backbone and major binding substrates are shown)



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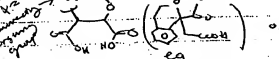
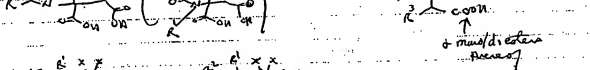
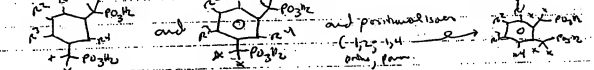


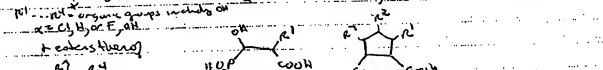
citric acid derivs.

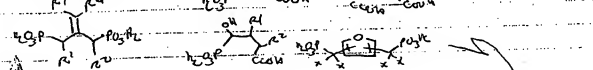
+ mono/di esters

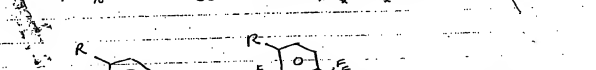


and positional isomers (1,2-, 1,3-, 1,4- only, form)









R = large lipophilic group to fill pocket specific to PTEN binding site

when X is oxygen that will bind to the Arginine found in the PTEN protein

IDEAS ON PTEN INHIBITORS!

I disclose herewith the concept of delivering PTEN inhibitor to the bone marrow cells for the purpose of preventing and/or inhibiting the bone marrow suppressing effects of chemotherapy and/or radiation therapy. Such inhibitors are envisioned to be attached to carriers to deliver them to the marrow. Carriers such as peptides, proteins & monoclonal antibodies are envisioned. Also envisioned are PTEN inhibitors modified covalently as prodrugs with attachive functionality to attach to such carriers. Also envisioned is PTEN inhibitors attached to bone seeking groups to deliver them to bone & allow for release to the nearby bone marrow cells to give a protective effect. I also envision incorporating PTEN inhibitors into polymers (covalently or physically entrapped) that are of a certain size to be targeted selectively to the marrow versus other organs.

All of the above approaches are designed to deliver PTEN inhibitors selectively to the bone marrow to protect the bone marrow during & after treatment from the bone marrow suppressing side effects of chemotherapy and/or radiation therapy.

Continued on Page

Read and Understood By

Joseph R Garl 4-1-02 Benjamin Nash 5/2/02

Signed Date Signed Date

26) Cooper, B.H, Garlich, J.R, Ritzler, S. "Solution Phase Parallel Synthesis of a 1408 Member Library of Phosphonic Acids and Esters, Poster presented at the 1st Annual Indiana ACS Poster Session, Indianapolis, Indiana, October 9, 2000.

Specific Aim 2: Subproject 2: **Identification of PTEN Inhibitors**

Background for PTEN. Cellular processes are to some extent controlled by cycles of phosphorylation and dephosphorylation involving lipids and proteins. PTEN (phosphatase located on chromosome 10) is a dual specificity phosphatase which dephosphorylates an important lipid second messenger, phosphatidylinositol 3,4,5 phosphate [Ptlins(3,4,5)P3] to control cell division and apoptosis. It is mutated at high frequency in human malignant disease (incidence varies from 20% to 95% depending on tumor type). Preliminary data from the Durden group has implicated PTEN in the control of tumor-induced angiogenesis and the control of immunoreceptor signaling suggesting that this is a major drug target for control of angiogenesis and inflammatory signals.

Other laboratories have shown that PTEN exerts control of sensitivity of cells to stress induced apoptosis. More recently the Durden group demonstrated the first direct evidence that

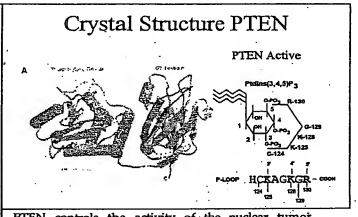


Figure 1. This shows crystal structure of PTEN with P-loop and amino acid sequence which composes the P-loop and its predicted interactions with the phosphoinositol ring. The 3 highly conserved basic residues in the P-loop, R, 130; K, 125 and K, 128 associate with the negatively charged phosphate groups in the phosphoinositol ring to coordinate catalysis at the D3 position mediated by conserved cysteine residue at position 124 which forms the critical thiol-phosphate intermediate required for catalysis [1].

PTEN controls the activity of the nuclear tumor suppressor protein, p53. This suggests that PTEN coordinates cell sensitivity to cell growth signals to balance cell proliferation with cell death (apoptosis) and angiogenesis.

These data together with evidence in the literature support the hypothesis that PTEN inhibition will modulate the apoptosis response under conditions of stress. Such inhibitors would increase PIP3 levels and prevent programmed cell death and promote survival of important cell populations cardiac myocytes, neuronal cells, etc under conditions of genotoxic or environmental stress e.g. hypoxia. Such inhibitors would serve to salvage tissue under conditions where stressful stimuli would tend to destroy normal organ functions. Therefore an agent which would inhibit PTEN thereby augmenting levels of PIP3 would be likely have therapeutic efficacy in a number of disease states associated with uncontrolled cell death and tissue damage. In particular we believe the delivery of such inhibitors to the bone and bone marrow could be a major advance in protecting marrow from the debilitating effects of chemotherapy and radiation therapy.

The crystal structure of PTEN has recently been reported with a tartrate molecule in the active phosphatase pocket [1]. The overall structure of PTEN is shown in Figure 1 along with the

structure of its substrate, PtIins(3,4,5)P3. The superimposition of phosphate groups at the D3 and D4 positions of Ins(1,3,4,5)P4 with those of the carboxylate groups of the tartrate found in the crystal structure is shown in Figure 2, reproduced from the literature [1]. The PTEN active site pocket is about 8 angstroms deep with an oval opening of about 5 by 11 angstroms. The left-side extension of the pocket in which the D5 phosphate protrudes is unique among phosphatases and thus represents a target space for us to fill in order to prepare specific PTEN inhibitors. In fact, mutations at Gly129 in the T1 loop decrease the size of this extension and disrupts PTEN's PI(3,4,5)P3 phosphatase activity but allow for retention of tyrosine phosphatase activity (because tyrosine would not require this extention pocket space)[2,3].

Chemistry Research Plan. Based on the crystal structure one aspect of our inhibitor design will be to prepare tyrosine-phosphate derivatives substituted such that they would indeed occupy this pocket extension space and hence be specific for PTEN and not tyrosine phosphatases. An additional difference between PTEN and other protein tyrosine phosphatases is the presence of two basic groups, Lys-125 and Lys-128, in the center of the P-loop (Figure 1,2). These basic groups (protonated underphysiological conditions) give rise to the attraction of the highly anionic PI(3,4,5)P3 substrate. This information thus serves as our second point of molecule design which is to include P-loop lysine binding moieties. These would include acidic groups such as carboxylate, phosphate, phosphonate, sulfonate, hydroxamate, phenolate, and the like which are capable of bearing an negative charge at physiological pH.

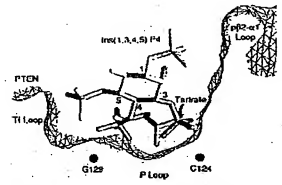


Figure 2. Model of Ins(1,3,4,5)P4 Binding to the PTEN Active Site. The phosphate groups at the D3 and D4 positions of Ins(1,3,4,5)P4 are superimposed on the crystal structure derived carboxylate groups of the tartrate molecule bound in the PTEN active site. Note the extended pocket unique to PTEN on the left side into which the D5 phosphate group protrudes.

The use of molecular modeling based on the crystal structure of PTEN is critical in getting us started on a focused combinatorial library approach to molecules that have the general required binding features but it should be noted that the crystal structure is a static picture. The actual solution dynamic structure of PTEN is unknown and probably not knowably for the foreseeable future. We thus will use combinatorial chemistry coupled with high throughput PTEN inhibition assays to probe the space around the binding site to find inhibitors and then to optimize such lead inhibitors. Thus, the crystal structure and molecular modeling are tools to get us in the general vicinity of chemical space and then combinatorial chemistry will allow us to zero in on good PTEN inhibitors.

While we are requesting resources to perform the intial guiding molecular modeling studies we have done some preliminary work to show that we can formulate two initial rational approaches to discovering PTEN inhibitors using UPS chemistry. We anticipate additional approaches being formulated upon initiation of project modeling studies. The intitial target library is is shown by the structure 3 in Figure 3. This template makes use of the fact that phosphotyrosine is a substrate for PTEN and hence structures such as 3 are expected bind with PTEN in the active pocket. However, the methylene group between the phenyl ring and the

phosphonate group creates a non-hydrolyzable bioisostere of phosphotyrosine. Thus structures such as 3 should bind in the active pocket of PTEN but will not be dephosphorylated and released. The functionality we introduce in the R1, R2, and R3 groups will be designed to fill the unique extended space of the binding pocket of PTEN to impart specificity to the inhibitor and also provide for additional binding interactions with the known groups in the PTEN binding pocket.

Briefly, the library synthesis shown in Figure 3 starts with commercially available Fmoc-Pmp-OH (1) which is then attached to solid-phase resin using coupling conditions minimizing reactions at the phosphonate group [4]. The Fmoc group is removed from the nitrogen using piperidine and the free primary amino group is made ready for UPS chemistry as described in Subproject 1, Figure 2. The UPS chemistry is perfomed as described for tandem dialkylation (di-UPS) introducing the first point of diversity R1 derived from a large set of electrophiles [5,6]. The nitrogen is then deprotected and reacted with a variety of acylating agents such as acid chlorides, sulfonyl chlorides, isocyanates, chloroformates and the like to introduce a wide variety of diversity group R2. It should be noted that the primary amine could also be reductively aminated prior to acylation to introduce yet another point of diversity on the nitrogen (instead of the NH present in 3). Lastly, the construct will be cleaved from the resin which in the case of Wang resin would lead to R3= -OH (carboxylic acid). Other resins can be used to introduce different functionality in R3. Alternatively, the Garlich group has pioneered a novel cleavage reaction that introduces additional functionality in the cleavage step using readily prepared aminimines as nucleophiles capable of cleaving the resin-ester linkage. Because of the potential for large numbers of compounds that can be generated with three points of diversity a considerable effort will go into choosing the inputs based on what is known about the aminoacid groups that make up the PTEN active site as well as the modeling of the binding site three dimensional space.

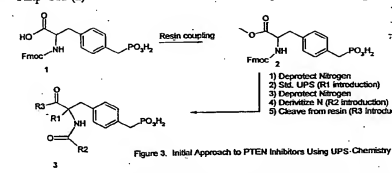


Figure 3. Initial Approach to PTEN Inhibitors Using UPS-Chemistry

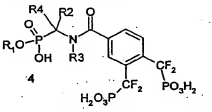


Figure 4. Proposed PTEN Library Based on Aminophosphonate UPS Chemistry

Another library of PTEN inhibitor targets, 4, is planned which will utilize the amino-phosphonate UPS chemistry to be worked out in Subproject 1. The aminophosphonate substitution pattern is to be prepared by methods developed in Subproject 1 allowing access to resin-bound intermediates such as 26 in Figure 6 of Subproject 1. These molecules will then be reductively aminated to introduce R3 and then acylated with a di-phosponate benzyl moiety to give a library represented by 4 in Figure 4. It should be noted that initially we will obtain racemic compounds from the UPS chemistry and if bioactivity is sufficient we will separate the stereoisomers or resynthesize the individual isomers using stereoselective UPS chemistry pioneered by the O'Donnell group. These molecules represted by 4 are designed to be non-hydrolyzable mimics of Ins(3,4,5)P3 and bind in the pocket similarly as shown in Figure 1 for

Ins(1,3,4,5)P4. In this case we have proposed the difluoromethyene-phosphonic acid group as the D3 and D4 phosphate mimics because such bioisosteres have recently been shown to be both isosteric and isoelectronic in character compared to the parent phosphate monoester [7]and would thus be expected to bind tightly. The three points of diversity are again aimed at binding to the other known amino acid residues in the binding pocket. The phosphonate half-ester group will serve as the mimic of the D5 phosphate monoester in Ins(3,4,5)P3 and the ester substituent (R1) of **4** is expected to project towards the Tl loop for additional binding interactions. Lastly, the 1,3,5 benzene ring substitution pattern is based on modeling which positions the two ring phosphonates near the lysines of the P loop (Figure 2) and allows the flexible backbone [(-C(O)N(R3)CHR2P(OH)(OR1)] of **4** to arch across to put the third phososphonate group near the Tl loop. Further refinements will be made based on bioactivity data to decrease flexibility and build a second generation rigid scaffold.

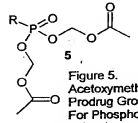


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Figure 5. Acetoxymethyl Prodrug Group For Phosphonates

Since PTEN inhibition is to take place inside a cell for therapeutic purposes there is a fundamental barrier to using small charged molecules (such as phophonates). However, there has been some work recently on using acetoxymethyl groups as a physiologically reversible protecting group to allow polyphosphonates to penetrate cell membranes [8]. This is shown as structure **5** in Figure 5. Thus, leads from both libraries described above can be converted to such phosphonate derivatives in order to get into cells to inhibit PTEN. However, there is still a delivery issue in that since PTEN is involved in cell signaling we need to introduce specificity in its targeted tissue or there would undoubtably be severe side effects from PTEN inhibition in nontarget tissue. The Durden and Garlich group are collaborating independently from this proposal on new methods to deliver cell signaling inhibitors to the skeleton. This is an excellent site to deliver therapeutic molecules to in a prodrug form such that as they are liberated from the bone the molecules are able to diffuse from the bone and migrate into the adjacent bone marrow and the cells therein. We are thus limiting the therapeutic effects to the bone and bone marrow as the target tissue. This is a large commercial market because there are numerous diseases of the bone and bone marrow to attack as well as aiming protectants from chemotherapy and radiation therapy to the dose limiting organ which is the bone marrow. We thus have devised a new approach to not only deliver agents to the bone but to design them such that they are hydrolyzed over time or under acidic conditions when osteoclasts resorb bone to liberate the free active molecule. We are proposing to combine this approach with the masking of the phosphonic acids technology to give the phosphonate derivative shown in Figure 6. In this manner we believe we can target polyphosphonates to the bone using the known bone seeking moiety **10** [9] attached through a readily acid cleavable bis-orthoester linker based on **8** [10]. Once at the bone the conjugate will initially cleave to yield **7** and the other molecules shown which are not expected to

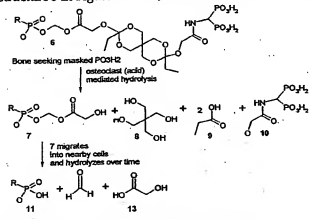

Figure 6. Schematic For Bone Seeking Masked Phosphonate Group (second identical ester group of phosponate omitted for clarity)

generate any problems in vivo. Then compound 7 can migrate into nearby cells and slowly over time hydrolyze to yield the active PTEN inhibitor 11 which will be trapped inside the cell. The number of oxygens in structure 6 is expected to lend desirable water solubility to the large conjugate.

We do not expect to optimize all of the above delivery and prodrug chemistry within the proposal period but we do expect to take at least one library PTEN inhibitor lead through this synthetic modification, demonstrate affinity to hydroxyapative (bone mineral), show acid mediated generation of 7, migration away from hydroxyapatite and entry into a cell whereupon it will be demonstrated to inhibit PTEN. This will be a proof of concept study designed to attract additional federal grant money to study this approach further.

Biological Evaluation Overview: Screening for PTEN inhibitors will occur in 5 different phases requiring positive results for a compound to be promoted from one assay phase to the next:

PHASE 1; High Throughput Bioassay. The combinatorial libraries will be screened for PTEN inhibition using purified recombinant PTEN phosphatase in a 96 well high throughput format using a colorimetric assay based on the detection of inorganic phosphate released from phosphopeptides or synthetic phospholipid substrates in vitro. Liberated inorganic phosphate is detected in twenty microliters of supernatant using the Malachite green assay and an inorganic phosphate standard curve. Malachite green reaction with inorganic phosphate is detected spectrophotometrically at 620 nm wavelength [11]. A series of endpoint reaction data points can be used to construct a kinetic curve representing PTEN enzymatic activity. The threshold for considering positive effect of candidate compounds for anti-PTEN activity will be 20% inhibition. This benchmark can be adjusted to give a manageable number of compounds to focus on. All compounds made will be put through this testing at the bioassay facility.

PHASE 2; PTEN Selectivity Bioassay. The hits from PHASE 1 screening will be further examined to determine if they possess the desired selectivity for PTEN inhibition versus other phosphatases. To determine specificity the Durden group will then evaluate these compounds for inhibitory effects on other protein phosphatases including the 5' phosphatidylinositol phosphatase, SHIP [19] and a number of recombinant protein tyrosine phosphatases under study in the Durden aboratory to include SHP-1, SHP-2, PTP1B and PTP [13-16]. Depending on how many hits from Phase 1 are being examined in phase 2 this may be a medium throughput assay to be performed in the bioassay facility. In this manner we hope to isolate small molecule inhibitors which target specifically the PTEN active site and not other lipid or tyrosine phosphatases.

PHASE 3; IC50 Determination. Compounds showing selectivity for PTEN inhibition will then be examined at various concentrations to determine IC50 values. Linearity for this assay is established for each screening run in the absence and presence of known phosphatase inhibitors. Positive controls for inhibitory activity will include oxidizing agents, vanadinium ions and phenylarsine oxide and para nitrophenylphosphate as competitive and irreversible inhibitors of phosphatase activity (17, 18). Inhibitory activity of candidate inhibitors will be compared to IC50 for these compounds in vitro.

PHASE 4; Functional PTEN Bioassays. The compounds with the best selectivity and best IC50 values will be evaluated for their ability to functionally inhibit PTEN in cell-based assays. The Durden group has an established cell-based assay based on an immunoreceptor signaling pathway in COS7 cells which is under the negative control of PTEN. The capacity to block PTEN would be measured by augmentation in immunoreceptor signaling as measured by increased Fc receptor mediated phagocytosis. This assay is easily and effectively adapted for medium throughput screening and measures the inhibitors ability to suppress PTENs capacity to inhibit phagocytosis in heterologous COS cell system [20]. Briefly, COS7 cells are cotransfected with cDNA constructs required for IgG mediated phagocytosis of opsonized sheep red blood cells in

presence of absence of PTEN cotransfection. PTEN transfection completely abrogates phagocytosis in this assay system. Therefore candidate inhibitors of PTEN will be tested in this system for inhibitory effects on PTEN, PI-3 kinase pathway and the capacity to reverse the 100% inhibitory effects of PTEN. The Durden group will also test confirmed PTEN inhibitors in this phase for capacity to block PTEN induced arrest in integrin directed migration in a well established PTEN reconstituted tumor cell line [19]. Briefly, the Durden group has been able to completely abrogate integrin directed migration in U87MG cells by the stable and inducible expression of PTEN. This migration system will be used as an additional cell based assay for inhibitors of PTEN. PTEN inhibition will induce augmented integrin directed migration in this genetically determined system of PTEN suppression. In addition, cell based functional assays which are regulated by SHP-1 are under study in the Durden laboratory and could serve to confirm specificity in cells. These cell-based assays are all available in the Durden laboratory [20]. It should be noted that at this stage there will be only a handful of the best compounds. In order to perform cell-based assays we will likely have to convert the phosponate containing PTEN inhibitors to the phosponate masked analog using a bromomethyleneacetoxy derivitizing reagent to give the nonionic prodrugs as shown in Figure 5.

PHASE 5; Bone Localized Bioactivation Bioassay. The final phase of analysis will involve conjugation of PTEN inhibitors to organic linker constructs as described in chemistry section (Figure 6 derivatization scheme) for targeting PTEN inhibitors to bone or bone marrow. The compounds will be tested in hydroxyapatite binding assays designed to establish the degree of binding activity for hydroxylapatite surfaces. The capacity of osteoclasts to liberate the parent PTEN inhibitor from bone matrix (hydroxyapatite) will be tested as will the ability of the liberated compound to still perform PTEN inhibition. These bone targeting and liberation studies are proof-of-concept and thus will not be exhaustive studies.

Summary of Objectives and Criteria for Success; Subproject 2:

1. Use molecular modeling to design and select potential library inputs using UPS chemistry to access chemical space based on the known crystal structure of PTEN
2. Use molecular modeling to design libraries using aminophosphonate UPS chemistry (developed in Subproject 1).
3. Develop a suitable throughput assay for PTEN inhibition and screen libraries.
4. The best compounds from 3 are tested for PTEN inhibition selectivity.
5. The best selective PTEN inhibitors from 4 are evaluated for IC50 values.
6. Evaluate best compounds (modified as acetoxymethyl derivatives) from 5 in cell based assays for functional PTEN inhibition.
7. Chemically prepare prodrugs that target the best compounds from 6 to bone (hydroxyapatite) and demonstrate reversibility in biological osteoclast assay (very limited compound set).

Path to Commercialization and Commercialization Potential: At the end of this project period we will have several validated PTEN inhibitor candidates. Additional funding will be obtained (SBIR, STTR, venture capital, etc) to perform the preclincal studies (toxicology, pharmacokinetics, efficacy in several animal models). Depending on how compelling the results are at this stage the technology may be partnered with a pharmaceutical company to assist in the development. In either case the timeline is about the same in that the next stage is a Phase I human clinical trial. Assuming a year for phase I, a year for phase II and then two years for Phase III human trial we plan for this technology to be available in a commercial drug within 10 years. The market potential for the products arising from this Subproject's research, for example a bone marrow sparing agent for application in chemotherapy, could be as much as $300 million per year. This estimate is based on the current success for related products such as Amgen's Neupogen (>$300

million in sales in 2000) for restoring neutrophils to adequate protective levels in patient undergoing chemotherapy. The potential advantage of our approach would be a single injection prior to chemotherapy versus daily treatment of patients with Neupogen. Additionally, with our approach the neutrophils should not even drop upon exposure to chemotherapy or radiation due to inhibition of PTEN induced apoptosis and thus the patients would not be at risk due to dropping neutrophils.

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Specific Aim 2: Subproject 2: Combinatorial Generation of DNA Recognition Metallopeptides.

Goals: The long-term objective of this subproject is to understand fundamental peptide-nucleic acid recognition phenomena with the aim of using the information obtained (1) to better understand the DNA/RNA recognition activities of naturally occurring nucleic acid binding agents and (2) to generate model compounds with efficient and selective nucleic acid binding properties that may also possess ability to design selective DNA binding agents with anti-cancer properties [1-5]. This research is being conducted through the study of structured and reactive metallo-peptides [6-7]. We have found that metallopeptides given the presence of natural amino acid side-chain functional groups coupled to their ability to cleave DNA via the redox activity of a metal center [8-9], can be significantly tight and the details of nucleic acid-ligand interactions that are not addressed by the many metal complexes or wholly organic structures that have been